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REVIEW

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Ovarian stimulation by promoting basal follicular growth

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Abstract

Background Most methods of ovarian stimulation rely on gonadotropin modulation. However, abnormal anti-Müllerian hormone concentrations are frequent in infertility, suggesting that defects in the gonadotropin-independent period of folliculogenesis preceding cyclic recruitment (i.e., basal follicular growth) may often occur. We need to better understand basal follicular growth and determine how to improve it.

Methods Section I summarizes a literature search concerning preantral and early antral folliculogenesis, cyclic recruitment, and selection. Section II presents current knowledge about interventions involving early antral folliculogenesis and cyclic recruitment.

Results While folliculogenesis following cyclic recruitment is gonadotropin-dependent, basal follicular growth is not. Basal follicular growth is regulated by follicle-stimulating hormone and local communication between the oocyte and its granulosa and thecal cells involving gap junctions and many autocrine/paracrine factors. This local communication sustains growth synergistically with follicle-stimulating hormone, but also suppresses this hormone to induce granulosa cell differentiation. As a follicle develops, its responsiveness to gonadotropin progressively increases. Section II describes 4 interventions affecting early antral folliculogenesis, including granulocyte colony-stimulating factor priming, bromocriptine rebound, carbohydrate metabolism intervention, and danazol priming, which have improved embryo development and live birth rate in patients with previous failures.

Conclusion Basal follicular growth modulation can increase live birth rates.

Keywords Preantral and early antral folliculogenesis, Cyclic recruitment, Assisted reproductive technology, Granulocyte colony-stimulating factor, Prolactin, Advanced glycation end-products, Danazol

Introduction

During the 45 years since IVF and ET first succeeded using a natural cycle, various methods of ovarian stimulation have been developed to increase pregnancy rate. These interventions, usually performed at about 2 weeks of follicular growth beginning at cyclic recruitment, almost exclusively modulate Gn control by administering Gn, GnRH agonist/antagonists, clomiphene citrate, and/or aromatase inhibitors. However, human follicular growth requires more than 6 months, and the early growth period preceding cyclic recruitment is particularly lengthy [1–6]. Moreover, many paracrine and autocrine factors act importantly in controlling folliculogenesis, especially before cyclic recruitment; after recruitment, Gn becomes pivotal [1–6].

Poor ovarian reserve and PCOS, respectively characterized by low and high serum concentrations of AMH, are encountered frequently in the clinical practice of ART. These conditions can interfere with obtaining high-quality oocytes using conventional ovarian stimulation. AMH is secreted mainly by preantral and early antral follicles before cyclic recruitment, so related major causes

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of ovarian dysfunction in these conditions probably act during folliculogenesis before cyclic recruitment [7]. Consequently, conventional ovarian stimulation has limited efficacy in patients with such impaired folliculogenesis before cyclic recruitment.

We need to explore new methods of earlier ovarian stimulation that improve folliculogenesis before cyclic recruitment. Among these, we serendipitously discovered that G-CSF administration enhances early antral follicle growth, improving embryos and live birth rates in patients with poor ovarian reserve [8]. In order to further advance the researches on earlier ovarian stimulation, first we need to know current knowledges on the regulations of early folliculogenesis, which are not so familiar to many physicians including me.

Section I of this review is an overview of early folliculogenesis including quiescent follicle activation, basal follicular growth, cyclic recruitment and selection, focusing especially on the mechanisms regulating them. Basal follicular growth is defined as the folliculogenesis from the activated quiescent follicle until the early antral selectable follicle just before cyclic recruitment [2] (See more in Fig. 1 and section I-1). The quiescent follicle is believed to be the primordial follicle in mice [1, 3] and the primordial, transitory, and small primary follicles in humans [2]. Section II describes treatment methods that promote basal follicular growth, aiming to provide insight and perspective on earlier ovarian stimulation.

Preantral and early antral folliculogenesis (Section I)

Details of preantral and early antral folliculogenesis have been increasingly clarified, but many early aspects remain controversial or less clearly known than subsequent events in antral folliclulogenesis. Extensive reviews of preantral and early antral folliculogenesis have been provided by several authors [1–6, 11]. Here I attempt to summarize quiescent follicle activation, basal follicular growth, cyclic recruitment, and selection, especially the mechanisms regulating them.

Overview of folliculogenesis (I-1)

Around the time of birth, oocytes are encapsulated by a single layer of squamous somatic pre-granulosa cells, forming primordial follicles [12]. Primordial follicles (including primordial, transitory, and small primary

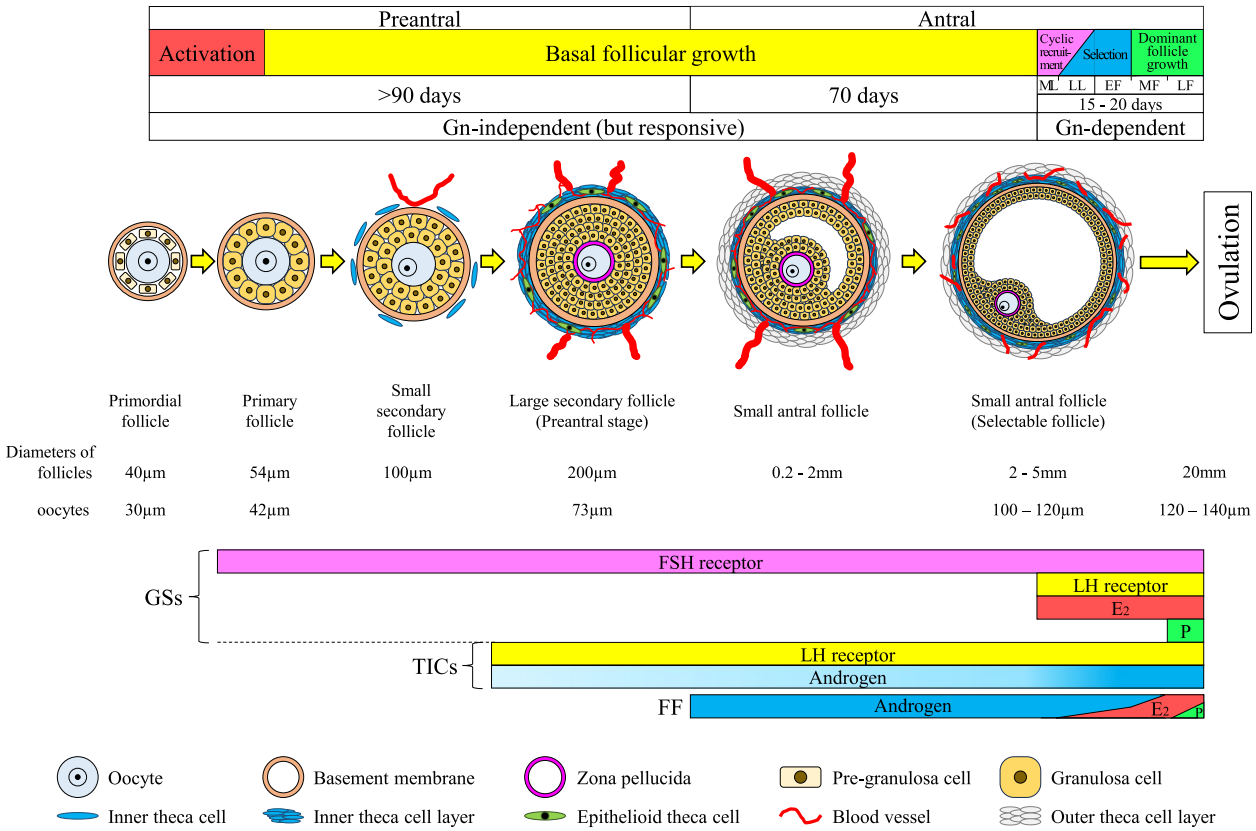

Fig. 1 Overview of folliculogenesis. This summary is based on Edson et al., 2009 [1], Gougeon et al., 2010 [2], Rimon-Dahari et al., 2016 [3], Hsueh et al., 2015 [4], Morton et al., 2023 [5], Gougeon A, 1986 [9], and Paulino et al., 2022 [10]

follicles in humans) [2] constitute the ovarian quiescent follicle reserve, from which only a selected subpopulation is activated and recruited into the pool of growing follicles that proceed to ovulation [1, 3]. According to current consensus, no primordial follicles are formed much later than birth, so this population of primordial follicles formed before and just after birth constitutes a fixed pool of ovarian reserve drawn upon until menopause [3]. However, in recent experiments involving old female rats, intraovarian transplantation of G-CSF-mobilized peripheral blood mononuclear cells recreated follicles at all stages including primordial follicles, restoring fertility [13]. Similarly, intravenous administration of bone marrow mesenchymal stem cells contributed to formation of primordial follicles in mice after cyclophosphamide-induced ovarian failure [14]. Existence of germline stem cells in adult mouse ovaries [15] suggests that while oogenesis in adult females is probably extremely limited under normal physiologic conditions, it might become possible after manipulations such as introduction of activated somatic oogenesis-supporting cells.

When a primordial follicle is activated, it grows into a primary follicle with a single layer of cuboidal granulosa cells (Fig. 1) [1–6, 11]. As these cells proliferate and the oocyte increases in size, the follicle becomes a small secondary follicle with 2 or more layers of granulosa cells. A small secondary follicle is supplied by one or two arterioles, terminating in an anastomotic network just outside the basal lamina [2]. At this stage, cells that form the theca interna begin to differentiate. Granulosa cells further proliferate, the zona pellucida is synthesized, and the theca interna with its vasculature fully differentiates, now representing a large secondary follicle. Some theca interna cells take on the appearance of steroid-secreting cells, termed epithelioid cells; at that point the large secondary follicle enters the preantral stage [2].

As the follicle continues to grow, fluid-filled pockets begin to form within the granulosa cell layers, eventually merging to form a single cavity called the antrum; the follicle has become an early antral follicle (Fig. 1). As fluid accumulates in the antrum and granulosa cells and theca interna cells proliferate, the follicle progresses to become a selectable follicle with a size between 2 and 5 mm [2]. In humans, selectable follicles may be seen at any time throughout the menstrual cycle, but their number and quality increase during the late luteal phase in response to increased concentrations of FSH, accompanied by regression of the corpus luteum [2, 16]. Selectable follicles are more receptive to cyclic hormonal changes, while earlier growing follicles are unresponsive [2, 17]. Among the recruited selectable follicles in the late luteal phase, the follicle destined to ovulate during the subsequent cycle is selected by the end of the early follicular phase [2, 9]. The selected follicle accelerates its growth, manifesting dominance and experiencing multistep preovulatory events after the LH surge, resulting in ovulation.

Folliculogenesis includes 5 main steps (Fig. 1): activation of quiescent follicles including primordial, transitory, and small primary follicles in humans [2]; basal follicular growth; cyclic recruitment of selectable follicles in the mid- and late luteal phase [9]; selection of follicle(s) destined to ovulate from among the recruited selectable follicles by the end of the early follicular phase [9]; and dominant follicle growth in the mid- and late follicular phase [2]. In humans, preantral folliculogenesis is estimated to take more than 90 days; early antral folliculogenesis up to selectable follicles, 70 days; and from cyclic recruitment of selectable follicles until ovulation, 15–20 days [2, 9] (Fig. 1). Advancing from the primordial follicle to the preovulatory follicle, oocytes and follicles respectively enlarge from 30 and 40 μm in diameter to 120–140 μm and 20 mm, respectively [10].

In humans, the early stages in folliculogenesis, from primordial follicles to selectable small antral follicles that represent basal follicular growth, do not depend on Gn, although they respond to it. In contrast, subsequent stages proceeding to ovulation are Gn-dependent [2]. This distinction is evident from observations that selectable small antral follicles can be seen despite congenital absence of bioactive FSH [18] and also in infancy, pregnancy, hypogonadotropic hypogonadism, and the post-hypophysectomy state, where concentrations of Gn are low [19]. In mice, where the preantral stage of large secondary follicles might be comparable to human selectable antral follicles, knock-out of *Fshβ* [20] and *Fshr* [21] genes does not stop folliculogenesis up to the secondary follicle stage but blocks it prior to antral follicle formation.

Basal follicular growth is regulated by interactions between FSH and local autocrine/paracrine factors produced by the oocyte, GC, and theca interna cells [2, 3, 5]. In addition, the Hippo signaling pathway constrains follicular growth [4, 11]. As a follicle develops, its responsiveness to Gn progressively increases [2, 5] and roles of Gn shift from selection of follicles for growth *vs.* atresia during basal follicular growth to growth, cell differentiation, and steroidogenesis after cyclic recruitment [5]. In humans, FSH receptor mRNA first can be detected in the GC of primary follicles [22], and LH receptor mRNA first can be detected in theca interna cells in the preantral stage of large secondary follicles [2, 23]. Although primordial follicles lack FSH receptor expression, possible indirect roles of FSH include formation of primordial follicles in the perinatal period and promotion of primordial follicle activation [5].

Follicular vascularization increases availability of oxygen, nutrients, and trophic factors such as FSH,

promoting follicular growth. VEGF participates not only in regulation of follicular angiogenesis but also direct regulation of follicular cell function [6]. In humans, VEGF expression first is detectable in both theca and granulosa cells of secondary follicles, increasing as follicles enlarge; VEGF receptors can be detected in follicular cells ranging from primordial to preovulatory follicles [6]. Mediated by its receptors on endothelial, thecal, and granulosa cells, VEGF promotes follicular angiogenesis and growth during primordial follicle activation, basal follicular growth, cyclic recruitment, selection, and dominant follicle growth [6].

Activation of quiescent follicles (I-2)

Inhibitory signals from the oocyte itself and signals from somatic cells maintain the ovarian quiescent follicle reserve, consisting of primordial follicles in mice and primordial, transitory, and small primary follicles in humans [2–4, 11] (Fig. 2). Activation of quiescent follicles is believed to be regulated in a gonadotropin-independent manner by paracrine signaling within the follicle and across the local environment [4, 11]. The balance of PI3K/AKT and mTOR cascades (activator) and the Hippo pathway (inhibitor) plays a major role in controlling the activation of quiescent follicles [11]. In addition, JAK/STAT, TGF-β, MAPK, and WNT signaling are thought as potential regulators [11].

Awakening signal activates mTORC1 in pre-GCs, stimulating their differentiations to secrete the Kit ligand [3, 11] (Fig. 2). The Kit ligand as well as various growth factors bind to the TK receptor and stimulate PI3K activity, increasing PIP_3. Then PDK1 is recruited and activated, phosphorylating AKT and S6K1. Although FOXO3 is preventing activation of the oocyte, activated AKT suppresses the inhibitory actions of FOXO3, resulting in activation of the oocyte [3, 4, 11]. PTEN converts PIP_3 to PIP_2, thus counteracting the action of PI3K [3, 4, 11].

Another target of AKT is the TSC1/2 complex-mTORC1-S6K1-rpS6 pathway [3, 11] (Fig. 2). AKT phosphorylates TSC2, destabilizing the TSC1/2 complex and releasing its inhibitory effect on mTORC1 [3, 11]. Activated mTORC1 stimulates S6K1-rpS6 signaling that promotes protein translation and ribosome biogenesis in oocytes [3, 11]. AKT also phosphorylates $p27^{Kip1}$, a cell-cycle inhibitor, opposing G1 arrest [3, 11].

The Hippo signaling pathway regulates organ size via control of cell proliferation, apoptosis, and stem cell self-renewal [4, 11]. It is regulated by the physical and mechanical microenvironment of cells in response to cues such as cell–cell contact, cell polarity, and energy stress [4, 11]. Hippo signaling consists of the

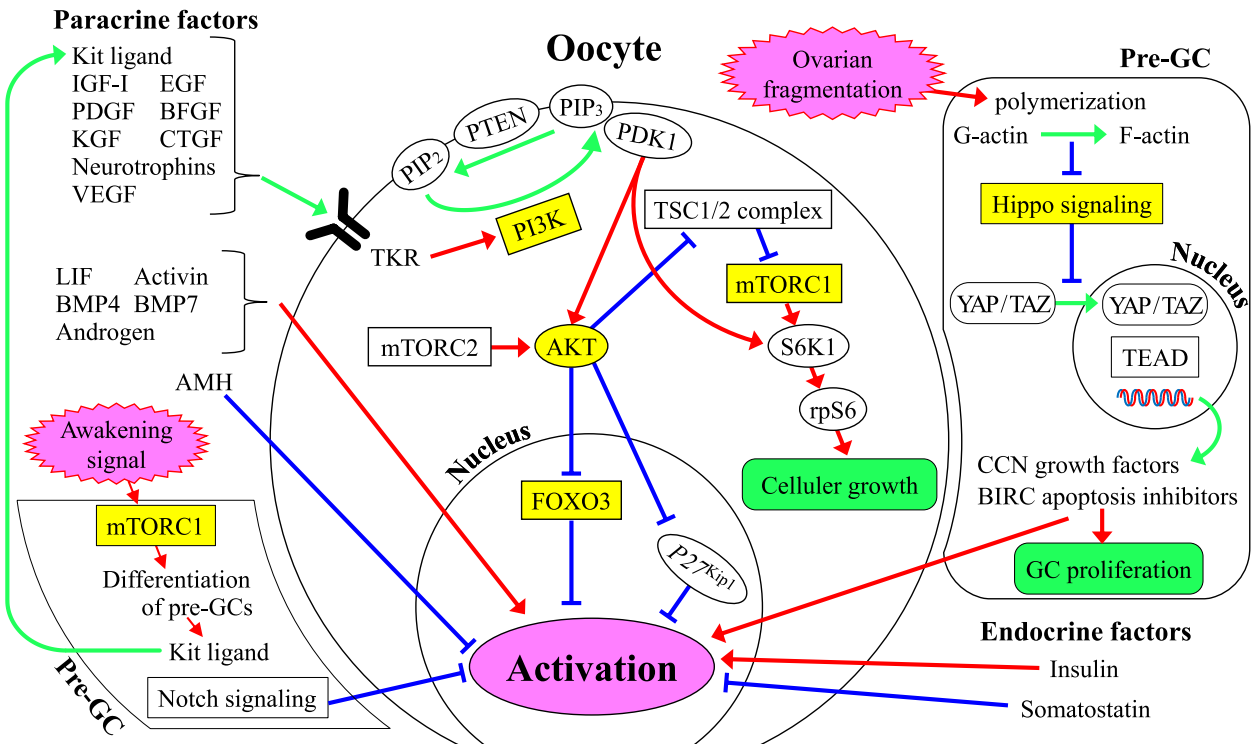

Fig. 2 Summary of activation of quiescent follicles based on Gougeon et al., 2010 [2], Rimon-Dahari et al., 2016 [3], Hsueh et al., 2015 [4], and Telfer et al., 2023 [11]

MST1/2-Sav1 complex and the LATS1/2-Mob1. Under basal conditions, the MST1/2-Sav1 phosphorylates the LATS1/2-Mob1, which in turn phosphorylates and inactivates downstream effectors, YAP/TAZ via their sequestration and proteolytic degradation in the cytoplasm [4, 11]. Upon the disruption of the Hippo signaling, unphosphorylated YAP/TAZ translocates into the nucleus and binds TEAD transcription factors, promoting the expression of CCN growth factor and BIRC apoptosis inhibitors. Thus, disruption of the Hippo signaling pathway stimulates activation of quiescent follicles, GC proliferation, and even later follicular growth [4, 11] (Fig. 2).

Furthermore, LIF, activin, BMP4, BMP7, androgen, and insulin stimulate activation of oocytes while AMH and somatostatin suppress it [2, 3]. Notch signaling in pregranulosa cells is involved in prevention of oocyte activation [3].

Regulation of basal follicular growth (I-3)

Basal follicular growth is regulated by interactions between FSH and local intra-follicular communication [2, 3, 5] but mostly is directed by local bidirectional communication between the oocyte and its granulosa cells and theca internal cells [2–5]. This bidirectional communication sustains follicular growth synergistically with FSH but also suppresses FSH to induce GC differentiation, according to developmental stage [2]. In addition, the Hippo signaling pathway, as described in the section of I-2, also constrains basal follicular growth according to various cues such as inter-follicular communications, follicular location within an ovary, and large structural changes associated with ovulation [4, 11].

Communication between the oocyte and its GC and TIC involves both direct cell-to-cell communication via gap junctions and autocrine/paracrine control (Fig. 3). In the former, the core protein that constitutes gap junctions, connexins 43 and 37, play pivotal roles [2, 3]. Autocrine/paracrine control involves many factors, including the TGF-β superfamily (TGF-β, BMP, GDF, activin/inhibin, and AMH), growth factors acting through TKR (kit ligand, EGF, KGF, HGF, BFGF, IGF-I, and various neurotrophic factors), androgen, R-spondin2, and CNP [2–5]. Oocytes express GDF9 [24], BMP15 [25], and R-spondin2 [26], all of which play central roles in cellular

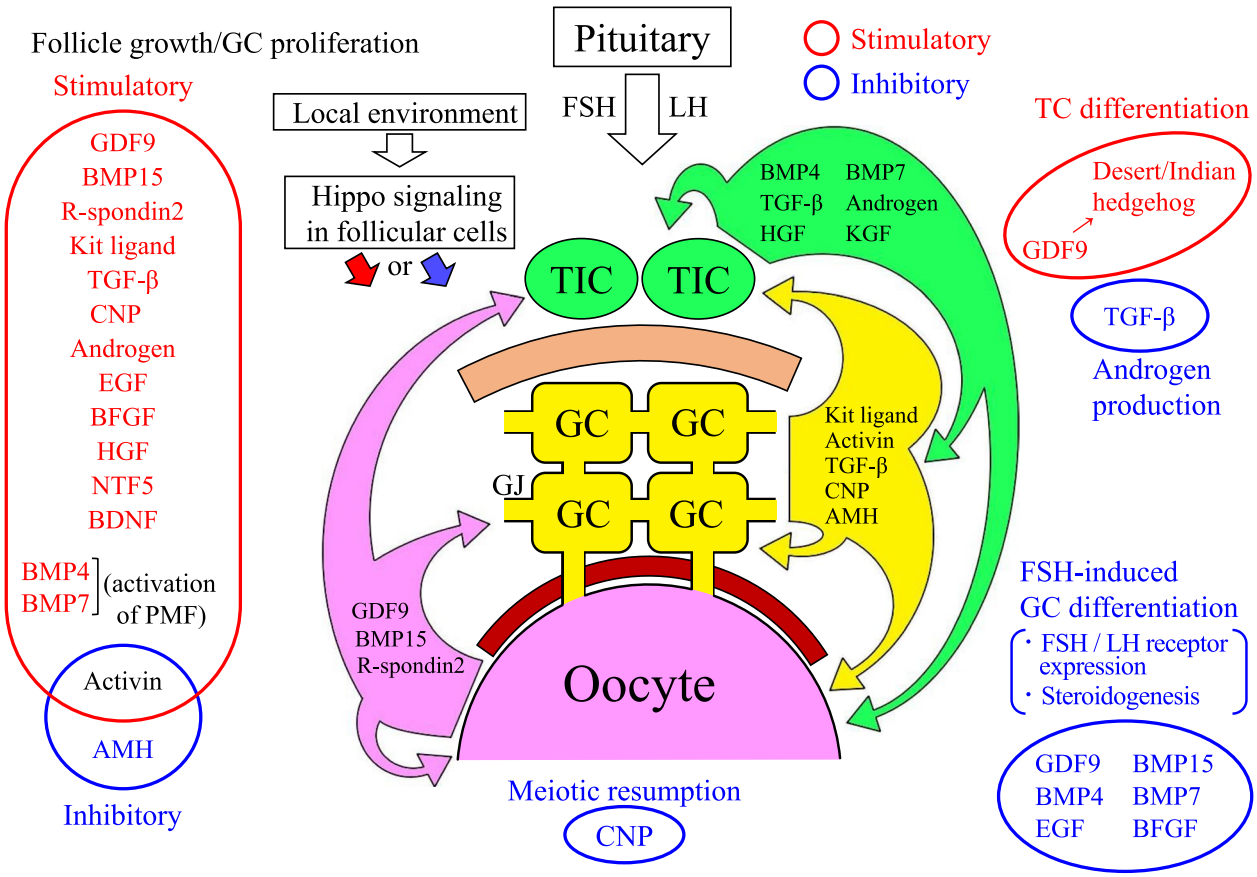

Fig. 3 Regulation of basal follicular growth, according to Gougeon et al., 2010 [2], Rimon-Dahari et al., 2016 [3], Hsueh et al., 2015 [4], Morton et al., 2023 [5], Shimasaki et al., 2004 [28], and Oktem and Urman, 2010 [31]. For additional references, see text

communication. GDF9-deficient mice exhibit a block in follicular development beyond the primary follicle stage, leading to complete infertility [24]. GDF9 administration in vivo enhances progression of primordial and primary follicles to small secondary follicles [27]. GDF9 stimulates inhibin production and basal estradiol synthesis in GC but inhibits FSH actions such as FSH-induced LH receptor expression and FSH-induced estradiol and progesterone synthesis [28]. GDF9 is required for granulosa cells to produce the paracrine signals of Desert hedgehog and Indian hedgehog, which in turn induce TIC differentiation [29]. BMP15 stimulates proliferation of granulosa cells in an FSH-independent manner [25], while naturally occurring point mutations of the BMP15 gene in Inverdale sheep (homozygous) and Hanna sheep cause infertility due to a block at the primary stage in folliculogenesis [30]. BMP15 inhibits FSH receptor expression in GC, resulting in suppression in FSH-induced LH receptor expression, progesterone synthesis, and activin/inhibin production [28]. R-spondin2 promotes development of primary follicles to the secondary stage in vitro and to the antral stage in vivo [26].

GCs produce kit ligand, activin, TGF-β, CNP, and AMH [2–4, 31]. Kit ligand promotes oocyte growth up to the preantral stage on GC monolayers [32]. BMP15 stimulates kit ligand expression in granulosa cells while this ligand inhibits BMP15 expression in oocytes, constituting a negative feedback loop that GC depends upon for mitotic regulation [33]. Additionally, kit ligand mRNA expression in granulosa cells is suppressed by GDF9 [34]. Activin and TGF-β promote preantral follicle growth in vitro [35]. FSH-induced growth of small secondary follicles, however, is inhibited by activin produced by large secondary/small antral follicles [36]. TGF-β suppresses androgen production in theca interna cells in most species [37]. CNP, secreted by GC of secondary and antral follicles, stimulates preantral and antral follicle growth [4]. In addition, in antral and preovulatory follicles, CNP increases cGMP in cumulus oophorus cells through its receptor, NPR2, as well as in oocytes by cGMP transfer through gap junctions [38]. Elevated cGMP concentrations in oocytes inhibit phosphodiesterase 3A, which increases intra-oocytic cAMP concentrations to maintain meiotic arrest [4, 38]. AMH inhibits FSH-stimulated preantral follicle growth [39]. In humans, AMH expression in GC is first detected in primary follicles, reaching its highest level in secondary follicles and small antral follicles 4 mm or less in diameter, and then gradually disappearing in larger (4–8 mm) antral follicles [40].

Theca interna cells produce BMP4, BMP7, TGF-β, androgen, HGF, and KGF [31]. BMP4 promotes survival of primordial follicles and stimulates primordial-to-primary follicle transition [41], while BMP7 promotes primordial-to-primary follicle transition and increases FSH receptor mRNA in the ovaries [42]. BMP4 and BMP7 modulate FSH signaling to promote estradiol production while inhibiting progesterone synthesis [43], acting as luteinization inhibitors such as BMP15 and GDF9 [28]. Androgen sustains early follicular development in monkeys [44] and simulates granulosa cell proliferation at all stages of development [45]. HGF and KGF stimulate granulosa cells to induce kit ligand, which in turn promotes the expression of HGF and KGF on theca interna cells [31].

EGF, BFGF, and HGF all stimulate granulosa cell proliferation in the absence of FSH, while EGF and BFGF inhibit FSH receptor mRNA expression and subsequent GC differentiation during early follicular growth [2]. NTF5 and BDNF are involved in progression from primary to secondary follicles [3].

Cyclic recruitment and selection (I-4)

When human follicles grow to become selectable small antral follicles (2–5 mm in diameter), they become responsive to cyclic changes of FSH and are recruited into gonadotropin-dependent further growth in response to increases in FSH, or alternatively undergo atresia [2, 46, 47]. After recruitment and especially after selection, growing follicles are believed to produce factors that suppress not only growth of less developed antral follicles [48] but also recruitment of following cohorts of selectable follicles [17]. These suppressive effects exerted by growing follicles appear to be much stronger than the FSH-stimulatory effect on recruitment because the number of small antral follicles 2 to 10 mm in diameter decreased even during maximal daily hMG stimulation in ART therapy, while the recruited cohort of follicles grew normally; the median follicle number at the beginning of hMG administration, 6 days later, and on the day of hCG administration respectively were 8 (interquartile range, 5–11), 1 (0–6), and 0 (0–2) [49]. Therefore, once a cohort of selectable follicles is recruited into further growth by an FSH rise, subsequent recruitment is blocked by the growing antral follicles despite the FSH elevation until the growing follicles ovulate or start to regress. Consequently, antral follicle development in the menstrual/estrous cycle occurs in waves over time [9].

Conventionally, a cohort of selectable follicles was thought to be recruited in the late luteal or early follicular phase once in each human menstrual cycle; subsequently, one of these ovulated after selection and further growth [9, 46]. However, multiple waves of antral follicle development have been observed during each estrous cycle in cattle (2–3 waves) [50, 51], sheep (3–4 waves) [52, 53], and other animals including horses, goats, llamas, buffalo, deer, and sub-human primates [47]. Each wave

is preceded a few days earlier by a transient increase in circulating FSH [50–52]. In an interovulatory interval (IOI), only the final follicular wave is ovulatory, while all preceding waves are anovulatory [51, 52]. In humans, 2 waves of antral follicle development were demonstrated by daily ultrasonographic observations in 68% of women and 3 waves in 32% [54]; a nadir in serum FSH occurred 3 days before emergence of any follicular waves [55]. In women with 2 follicular waves, a first anovulatory wave emerges at the time of ovulation, followed by emergence of a second ovulatory wave during the early follicular phase; in women showing 3 waves, a first anovulatory wave similarly emerges at the time of ovulation, with a second anovulatory wave emerging during the mid- to late-luteal phase (earlier than in women with 2 waves), and a third ovulatory wave emerges in the early to mid-follicular phase [47, 54]. The interwave interval (IWI) between second and third waves in women with 3 waves is significantly shorter than all the other IWIs in women with 2 or 3 waves [55]. Notably, the third wave emerges before the second wave has regressed [56]. Considering that recruitment occurs despite strong suppressive effects from preceding growing follicles, a recruitment stimulatory factor in addition to FSH may be at work in the late luteal to early follicular phase, possibly at the time of luteal regression. Disappearance of recruitment-suppressive effects of the corpus luteum does not fully account for this phenomenon, considering that in sheep small FSH surges and corresponding anovulatory follicular waves occur during the active luteal phase; neither FSH surges nor waves are suppressible even by administration of progesterone in the active luteal phase, indicating that the suppressive effect of the corpus luteum is less potent than the stimulatory effect of FSH [52]. As I suggest further on in section II-2, the unknown stimulatory factor might be an increase in prolactin during the late luteal phase.

For more than a decade, random start ovarian stimulation (RSOS), in which stimulation is initiated regardless of the day or phase of the menstrual cycle, has been successfully used in oncofertility and general infertility practice [56, 57]. Compared with conventional stimulation initiated at the early follicular phase, RSOS yields a similar number of oocytes with similar reproductive competence, resulting in similar rates of pregnancy and live birth after cryopreserved embryo transfers [56, 57]. Recruitment of selectable follicles thus appears fundamentally possible on any day of a menstrual cycle. However, RSOS tends to require longer stimulation and more Gn [56, 57], suggesting some differences from physiologic recruitment in the late luteal to early follicular phase. Consistently, the GC mitotic index of selectable follicles [9] and follicular responsiveness to exogeneous FSH in terms of GC proliferation [17] and aromatase activity [16] greatly increases in the mid- to late-luteal phase even though the antral follicle count (2–5 mm) does not differ significantly between cycle phases [58].

Selection is the final adjustment of the cohort of recruited growing follicles to the ovulatory quota, which is the species-characteristic number of follicles that ovulate in each cycle [2, 59]. Selected follicles are likely to be more sensitive to FSH [60], possibly because of enhanced FSH receptor expression or increases in local growth factors that augment FSH responsiveness [46]. Selectable follicles contain high concentrations of TGF-α, EGF, IGFBP, and activin [2, 19]. TGFα and EGF inhibit FSH-induced synthesis of estradiol and stimulate GC proliferation, IGFBP inhibits IGF-II to induce aromatase activity in GC, and activin stimulates GC proliferation and expression of FSH and LH receptors [19]. Consequently, GC of selectable follicles respond to FSH by proliferating, but not estrogen production, maintaining high androgen and low estradiol concentrations in follicular fluid [2]. In selected follicles, however, both proliferation and differentiation stimulators are influencing their GC, resulting in substantially increased follicular fluid estradiol concentration [2].

During selection, the IGF system plays a key role [2]. IGF-II increases in GCs of dominant follicles at that time [19]. Simultaneously, FSH stimulates an IGFBP-4 protease, PAPP-A, which cleaves IGFBP-4, subsequently increasing bioavailability of IGF-II. The increased free IGF-II stimulates both proliferation and steroidogenesis in GC and TIC, increasing estradiol and androgen [19], as well as differentiation of LH receptors on GC [2]. These effects are strongly enhanced by FSH and LH [19].

An orderly transition in the follicular environment from inhibin B and activin to inhibin A and follistatin appears important for dominant follicle development [47]. Follicular fluid inhibin B peaks at a follicular diameter of 9 to 10 mm, a time of divergence between dominant and subordinate follicles [47]. This is followed by an increase in inhibin A, which strongly enhances IGF- and LH-induced androgen production in TIC [2, 19, 47]. The androgen is then converted to estradiol by aromatase activity in GC [1], which can be detected in follicles of 10 mm or larger [2], enhanced by the increases in IGF-II. Activin inhibits LH-induced progesterone production by GC in preovulatory follicles [19], while follistatin has much higher binding affinity for activin than inhibin, so follistatin's net effect is facilitation of LH-induced progesterone production by GC in preovulatory follicles by suppression of activin effect [19].

Thus, estradiol and inhibin produced by the dominant follicle(s) suppress pituitary FSH release during the mid-follicular phase. As a result, other less developed antral

follicles are deprived of sufficient FSH stimulation for survival [46]. Dominant follicles, however, can maintain development despite decreasing FSH given their greater GC numbers, higher expression of FSH and LH receptors on GC, greater free IGF-II, and richer vascularization [2, 3, 19, 46, 47].

Further, concentrations of TGF-α and EGF in follicular fluid decrease during selection [19]. AMH expression in GC is greatest in secondary follicles and small antral follicles 4 mm or less in diameter, followed by gradual disappearance in larger antral follicles measuring 4–8 mm [40]. This AMH disappearance coincides with time of selection, but precise roles of AMH in dominant follicle selection are not known [47].

Interventions affecting basal follicular growth (Section II)

Although various interventions affecting basal follicular growth have been proposed to benefit patients with poor ovarian response to conventional ovarian stimulation, their effectiveness is unclear or doubtful, especially in terms of live birth [61]. Recently I serendipitously discovered that administration of G-CSF preceding conventional ovarian stimulation (G-CSF priming) increases AMH and improves embryo quality and live birth rate in patients with poor ovarian reserve [8]. In this section, I first describe G-CSF priming, and then 3 other strategies favoring basal follicular growth that I have been using successfully in my practice of ART for over 30 years. Finally, I discuss other previously reported methods.

In our studies, IBM SPSS Statistics (IBM, Tokyo, Japan) was used for statistical analyses. Data were tested for normality by the Shapiro–Wilk test. If data were not normally distributed, analysis was performed using the Mann–Whitney *U* test, the Wilcoxon matched-pairs signed rank test, or Spearman correlation analyses as appropriate. If data were normally distributed, unpaired *t* tests, paired *t* tests, Pearson correlation analyses, analysis of variance (ANOVA), or Fisher' s protected least significant difference (PLSD) test were performed as appropriate. Data also were analyzed using the chi-squared test, Fisher's exact test, multiple logistic regression analysis, receiver-operating characteristics (ROC) curve analysis, or discriminant analysis as was suitable. *P* values less than 0.05 were considered to indicate significance. Results are presented as the mean ± standard deviation (SD), except for results of the bromocriptine-rebound method which are presented as the mean ± standard error measurement (SEM).

G-CSF priming (II-1)

About 10 years ago, we administered G-CSF to overcome RIF in 10 patients with diminished ovarian reserve. While none of them achieved pregnancy by these ET, 3 of them conceived spontaneously 2 cycles later; one of these pregnancies involved twins in a 45-year-old woman without ovulation induction. We therefore suspected that G-CSF administration stimulated preantral or early antral follicle growth to improve ovulation 2 cycles later, resulting in pregnancies. We therefore initiated a prospective randomized controlled trial in patients with poor ovarian reserve to examine whether G-CSF priming preceding ART increased serum AMH and improved embryo development and pregnancy rate [8].

G-CSF (100 μg of lenograstim; Neutrogin, Chyugai Pharmaceuticals, Tokyo, Japan) was administered subcutaneously in the early luteal phase (2–5 days after the ovulation) of 2 consecutive cycles preceding ART (Fig. 4A). Ovulation was determined, based upon basal body temperature records, vaginal ultrasonographic findings (collapse of the dominant follicle), and, if necessary, serum progesterone determinations (1.5–3 ng/mL of serum progesterone was considered as the value on the ovulation day). We referred to this treatment as G-CSF priming. Ovarian stimulation using the long protocol and ART followed priming.

One hundred patients with poor ovarian reserve (AMH < 2 ng/mL, 20 to 42 years old) were enrolled and prospectively randomized into G-CSF or control groups of 50 patients each. Baseline patient characteristics were similar between G-CSF and control groups: ages, 36.6 ± 3.8 and 37.5 ± 3.5; BMI, 20.9 ± 2.3 and 21.1 ± 2.8; infertility duration, 2.3 ± 2.1 years and 2.4 ± 3.1; gravidity, 0.8 ± 0.8 and 1.0 ± 1.1; parity, 0.5 ± 0.6 and 0.4 ± 0.6; AMH, 0.98 ± 0.54 ng/mL and 0.91 ± 0.49; and similar prevalence of infertility causes (Refer more details to the reference [8]).

Both groups initially underwent conventional infertility treatment for 2 consecutive cycles, in which the G-CSF group additionally received G-CSF priming. Then each group underwent one cycle of IVF/ICSI and fresh ET. If fresh ET failed, cryopreserved ET was performed until live birth or embryo depletion. Serum AMH was measured before and after G-CSF priming in the G-CSF group, with similar measurements in the control group at similar time points.

Fertilization rate, embryonic development, implantation rate by fresh ET, and oocyte developmental competence were significantly improved by G-CSF priming (Fig. 4B and C). Clinical and ongoing pregnancy rates using IVF/ICSI-fresh ET were significantly higher with G-CSF priming (30% and 26% of 47 ART patients; 3 patients delivered with conventional treatment) compared with controls (12% and 10% of 49 ART patients; 1 dropped out). Significantly more G-CSF patients achieved cryopreservation of redundant blastocysts than

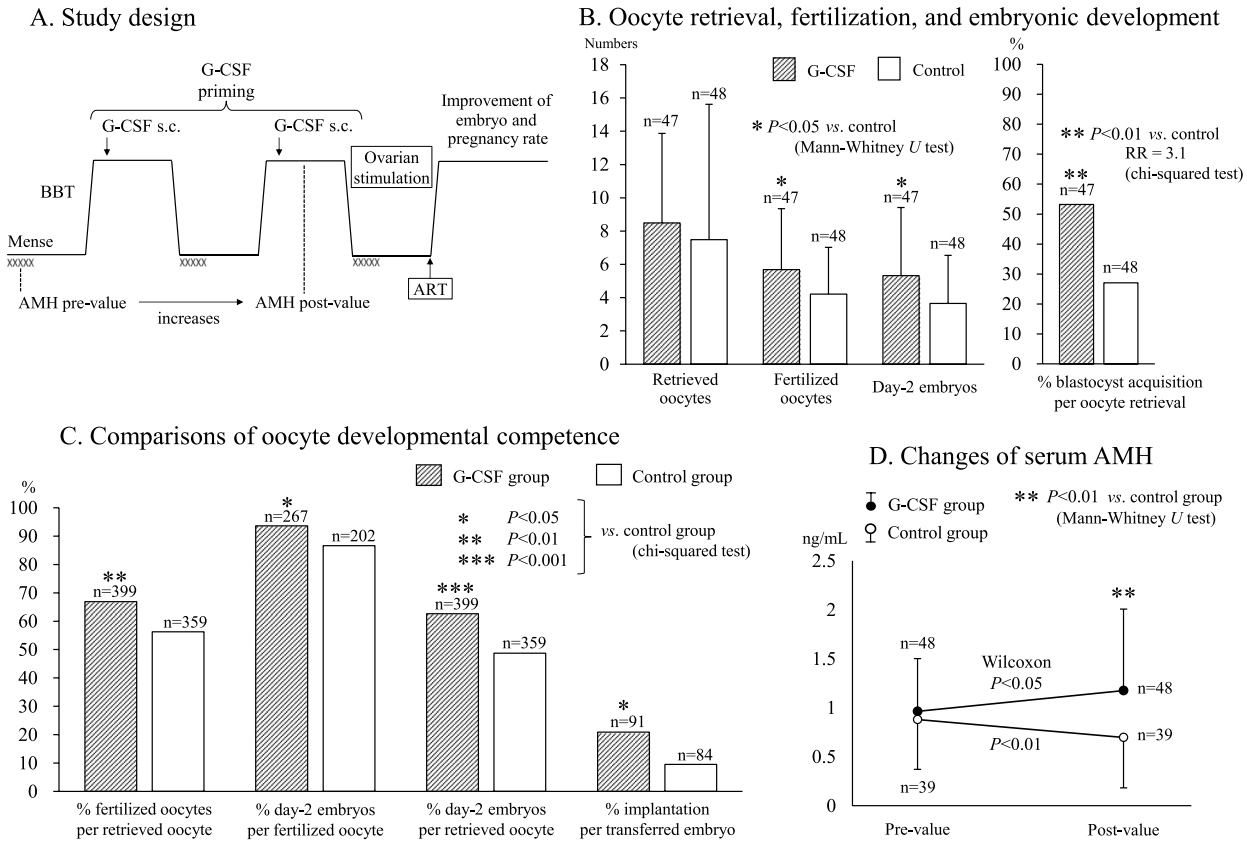

Fig. 4 G-CSF study design (**A**). Oocyte retrieval, fertilization and embryonic development (**B**), and oocyte developmental competence (**C**) were compared between G-CSF and control groups. Significantly more fertilized oocytes and day-2 embryos, a higher rate of blastocyst acquisition, and higher embryo quality were obtained in the G-CSF group. Implantation rate per transferred embryo was defined as (number of gestational sacs / number of transferred embryos) × 100%. Serum AMH significantly increased after G-CSF priming, while in controls AMH decreased, resulting in higher final concentrations of AMH in the G-CSF group (**D**). G-CSF, granulocyte colony-stimulating factor; AMH, anti-Müllerian hormone; BBT, basal body temperature; ART, Assisted reproductive technology. Revised from Jinno et al., 2023 [8]

controls (53% *vs.* 24%) The cumulative live birth rate was 32% in 50 patients with priming, significantly higher than 14% in 49 controls (relative risk, 2.8; 95% confidence interval, 1.04–7.7). Infants born after priming had no congenital anomalies, while infant weight, birth week, and Apgar score were similar between groups. Priming significantly increased serum AMH (Fig. 4D), suggesting enhancement of early antral folliculogenesis. No adverse effects of priming were observed. No significant differences in killer-cell immunoglobulin-like receptor (KIR) genotype were observed between patients in the G-CSF group with and without achievement of clinical pregnancy. G-CSF administration together with ET has been shown to overcome RIF most effectively in patients lacking 2DS1, 2DS5, and 3DS1 [62]. Accordingly, the mechanism by which G-CSF priming improves early antral folliculogenesis is likely to be unrelated to alleviation of RIF.

In diabetic rats, G-CSF administration consistently decreased ovarian follicular degeneration as well as degeneration and fibrosis of ovarian stroma, while increasing serum AMH [63]. G-CSF administration also significantly increased ovarian preantral follicles and serum AMH in rats with DOR induced by cisplatin [64].

Improvement of early antral folliculogenesis by G-CSF could involve various mechanisms. G-CSF has been reported to act directly against apoptosis [65–67], inflammation [63, 67, 68], and oxidation [63, 67] while favoring angiogenesis [67, 69] and growth promotion [67, 70]. Autocrine or paracrine influences on folliculogenesis by G-CSF [71] might be involved, considering that embryos derived from follicles with higher G-CSF were reported to implant more readily [72].

G-CSF also promotes egress of bone marrow stem cells (BMSC) into peripheral blood [73], which could aid tissue regeneration considering that ovarian transplantation of autologous BMSC collected by apheresis after G-CSF administration for 5 days was found to improve follicle and oocyte quantity, making pregnancy possible in poor ART responders [74]. On the other hand, human

plasma derived from apheresis after daily administration of G-CSF for 5 days, enriching it in BMSC-secreted factors, also improved follicular development and fertility in a mouse model of chemotherapy-induced ovarian damage [75]. Further, human umbilical cord mesenchymal stem cell-derived conditioned medium (hUCMSC-CM) opposed granulosa cell apoptosis and depletion of primordial follicles in cisplatin-treated mice [71]. Thus, an indirect mechanism involving BMSC-secreted factors might be responsible rather than transdifferentiation of BMSC.

Many previous studies have shown that G-CSF improves endometrial receptivity in the same luteal phase as it is administered. G-CSF Administration together with ET was found to increase implantation rates and clinical pregnancy in ART patients with repeated implantation failure or endometrial thinning [76–81]. G-CSF also reduced miscarriage rate and increased live birth rate in women with unexplained recurrent miscarriages [82]. In the absence of 3 activating KIR genes detected particularly frequently in women with unexplained recurrent miscarriage (i.e., lack of 2DS1, 2DS5, and 3DS1) [83], G-CSF has shown high effectiveness in overcoming repeated implantation failure [62]. Intrauterine administration of G-CSF was found to increase endometrial thickness in women with endometrial thinning [84]. Considering such observations, G-CSF administration in the early- and mid-luteal phase may improve endometrial receptivity by immunologic interactions and endometrial growth promotion.

In our present study, however, G-CSF was administered in the cycles preceding to ART and never in the ET cycles. Thus, the G-CSF direct effects on endometrial receptivity, as described above, is not likely involved in underlying mechanisms improving clinical pregnancy rate in our study. Consistently, we observed no effects of G-CSF priming on miscarriage rates or any association of G-CSF efficacy with KIR genotype. Furthermore, the significant increase in serum AMH and improvement of follicular and embryonic developments in our G-CSF group strongly suggest early antral follicle growth enhancement as an underlying mechanism.

Recently, a confirmation of our experience with G-CSF priming was reported in the annual meeting of Japan Society for Reproductive Medicine on November 14, 2024, showing that 3 of 8 women with AMH < 2 ng/mL and previous ART failures conceived with G-CSF priming [85].

Bromocriptine-rebound method (II-2)

While gynecologists recognize prolactin (PRL) as a hormone important in lactation and hyperprolactinemic anovulation, its biologic actions are far more varied. PRL, which evolved from the same ancestor hormone as growth hormone, is expressed in all vertebrate species studied, and has more than 300 diverse effects affecting reproduction and lactation, water and electrolyte balance, growth and development, metabolism and endocrinology, brain and behavior, immunologic control, and ectodermal integrity [86, 87]. In addition to the pituitary, PRL is also produced and secreted at many other sites (extrapituitary PRL), including follicles and endometrium [88]. PRL receptor (PRLr) is also expressed nearly everywhere in the body [87]. Following hypophysectomy in rats, PRL bioactivity initially decreased to 15% of normal but then rose to 50% because of a compensatory increase in extrapituitary PRL production; while simultaneous administration of anti-PRL antibody kept PRL at 0%, culminating in death of all treated rats [89]. This establishes PRL as a growth factor essential to growth and function of cells, acting in endocrine, paracrine, and autocrine manners. Provision of PRL, so highly important for tissues, appears to be carried out by both central (pituitary) and local (extrapituitary) production.

A midcycle PRL surge in serum and PRLr in GCs were demonstrated in humans [90], and abnormal follicular development was observed in a woman with isolated PRL deficiency [91]. These observations suggest that PRL plays a physiological role in human folliculogenesis. In human IVF, high concentrations of PRL in follicular fluid were associated with maturation of the oocyte-cumulus complex, successful fertilization, and pregnancy [92]. Hypoprolactinemia induced with continuous administration of bromocriptine was associated with lower rates of fertilization [93, 94] and embryo cleavage [94]. Therefore, PRL appears to play a stimulatory role in the growth and maturation of oocytes. Pathological hyperprolactinemia is well known to have detrimental effects on various ovarian functions [95]. Thus, appropriate levels of PRL are probably essential to the normal growth and maturation of oocytes.

About 33 years ago, malfunction of a PRL analyzer in our hospital made us misdiagnose normoprolactinemia as hyerprolactinemia; we erroneously administered bromocriptine to affected patients for 1 month, until discovery of the malfunction. After bromocriptine was discontinued, some of those patients conceived despite a long prior interval of treatment failure. In that manner we discovered that ovulation can improve after cessation of PRL suppression by bromocriptine [96]. We confirmed effectiveness of this novel method for ovarian stimulation, bromocriptine-rebound (BR), in a prospective randomized controlled trial [97]. The BR method is the same as the long protocol using a GnRH agonist and hMG, except that bromocriptine (Parlodel, Sandoz, Tokyo, Japan) is administered orally at a dose of 2.5 mg/

day from the early follicular phase (day 3–5) of the preceding cycle until 7 days before initiation of hMG administration (Fig. 5A). In practice, the day of bromocriptine cessation was scheduled as follows: when GnRH agonist in the long protocol was started on the luteal day 4–6, the following onset of menstruation was estimated according to the BBT chart by assuming the luteal phase to continue for a total of 14 days. Then, the day of hMG initiation was scheduled on the estimated menstrual day 3 and the last administration of bromocriptine was scheduled 7 days before the day of hMG initiation. Even if actual menstruation started earlier than estimated, hMG was started on the scheduled date, adhering to 7 days after bromocriptine cessation so that the actual cycle day of hMG initiation became a few days later than day 3. When actual menstruation started later than estimated in fewer patients, however, hMG initiation was delayed until the actual cycle day 3 so that the intervals between the bromocriptine cessation and hMG initiation became a few days longer than 7 days. Empirically, the efficacy of BR method is the same when the interval between bromocriptine cessation and hMG initiation is 7 to 14 days, while efficacy appears to decrease when the interval is less than 3 days.

Endocrinologically normal ovulatory women with at least one unsuccessful ART attempt using the long protocol were assigned at random to receive either the BR method or a typical long protocol. BR significantly improved fertilization and embryonic development, surpassing rates of pregnancy and delivery attained with repeated long-protocol treatment (Fig. 5E). In initial ART attempts using the long protocol, serum PRL concentrations during hMG administration (Fig. 5B) and PRLr mRNA expression in granulosa cells (Fig. 5D) were significantly higher in non-pregnant than pregnant patients. In the BR method, serum PRL concentrations fell significantly with bromocriptine treatment but increased significantly beyond the pretreatment value after discontinuation of bromocriptine, showing a rebound phenomenon (Fig. 5C). Subsequently, when ART was repeated

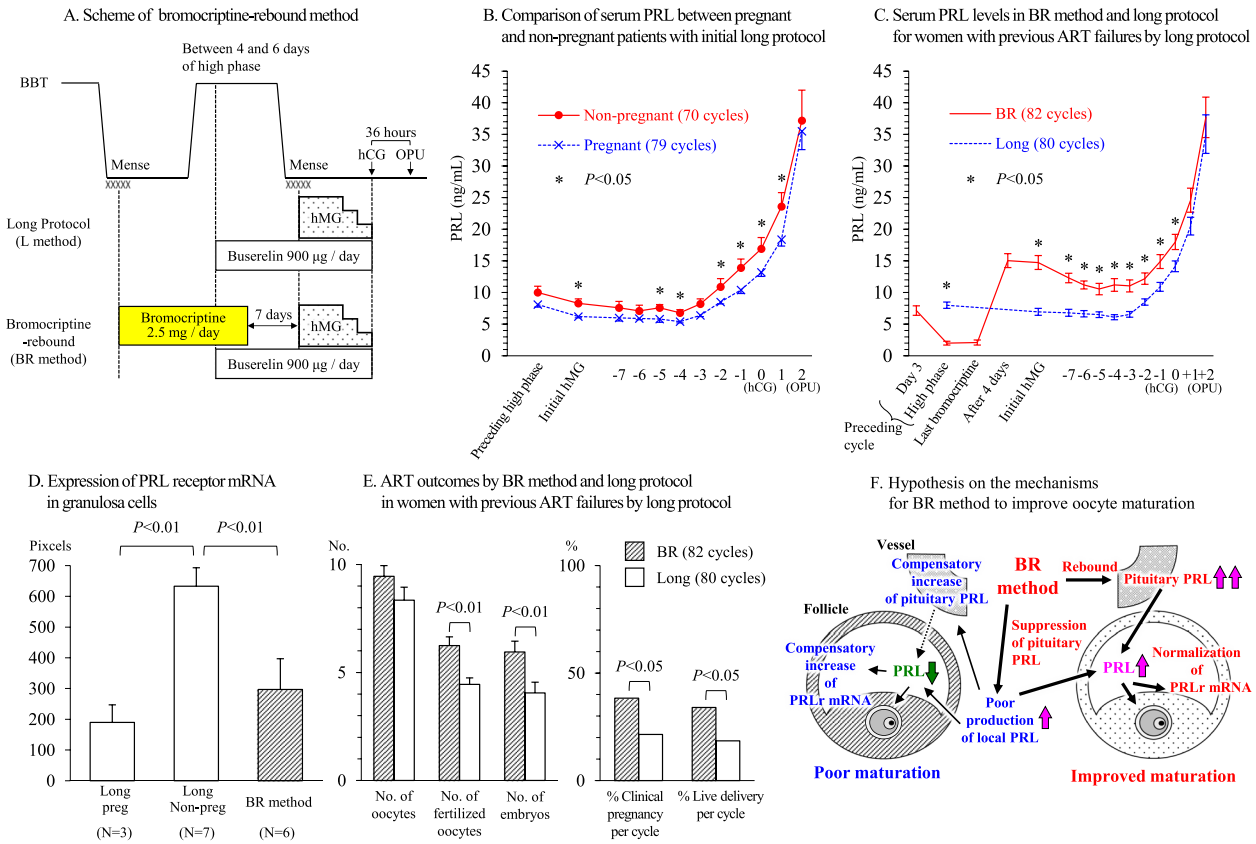

Fig. 5 The bromocriptine-rebound (BR) method is the same as in the long protocol, except that bromocriptine is administered from the early follicular phase of the preceding cycle until 7 days before hMG administration (**A**). In the initial ART with the long protocol, serum PRL during hMG administration (**B**) and PRLr mRNA expression in granulosa cells (**D**) were higher in patients not achieving pregnancy. When ART was repeated with the BR method in the non-pregnant patients using the initial long protocol, serum PRL during hMG increased further (**C**), and PRLr mRNA expression in granulosa cells decreased (**D**). The BR method improved fertilization, embryo development, and the rates of pregnancy and delivery (**E**). Hypothesized mechanisms for the BR method in improving oocyte maturation (**F**). OPU, oocyte pick-up. Revised from Jinno et al., 1997 [97]

using the BR method in non-pregnant patients using the long protocol, serum PRL concentrations during hMG stimulations increased further (Fig. 5C) while PRLr mRNA expression in granulosa cells decreased (Fig. 5D).

As an explanation of these results, we propose that patients with poor ART outcomes using the long protocol may have decreased follicular PRL production and/or postreceptor responsiveness of GC to PRL, causing compensatory increases in serum PRL concentrations and GC PRLr mRNA and PRLr (Fig. 5F) [97]. Most likely, however, this compensation is inadequate, so that oocyte maturation is impaired due to inadequate growth-stimulatory effect of PRL. Bromocriptine suppresses pituitary PRL production but has no effects on extrapituitary PRL production [88]. Therefore, when the BR method is used in such patients with poor ART outcomes using the long protocol, bromocriptine administration suppresses pituitary, but not follicular, PRL production during the cycle preceding to ART cycle. Small antral follicles growing there are compelled to adapt themselves to hypoprolactinemic circumstances by increasing their follicular PRL production and/or postreceptor responsiveness of GC to PRL to compensate decreased PRL supply from pituitary. After discontinuation of bromocriptine, the serum PRL concentrations increase beyond the pretreatment value as a rebound phenomenon by cessation of suppression. Consequently, recruited antral follicles, which are improved of follicular PRL production and/or responsiveness to PRL, receive increased pituitary PRL supply during hMG stimulation, resulting in an improvement of oocyte maturation by adequate PRL effects. A decrease in PRLr mRNA expression appears to reflect the recovery of follicular PRL production and/or postreceptor responsiveness to PRL.

PRL production by human endometrium has been demonstrated, typically following day 23 of the menstrual cycle [98, 99] and occasionally as early as day 18 [99]. Endometrial PRL production increases as decidualization progresses [98, 100], showing more rapid increases as implantation progresses [101]. Similarly, PRL is detectable in endometrial fluid from monkeys on day 24, followed by a rapid increase in secretion [102]. Increased endometrial PRL could reach the ovary by diffusion or via blood vessels and lymphatics, increasing PRL concentrations in mid- and late luteal phase follicles, which might stimulate cyclic recruitment as mentioned in section I-5. I recently attempted to increase PRL concentrations in the late luteal phase by direct pharmacologic intervention, resulting in beneficial effects on oocyte developmental competence similar to those with the BR method that were followed by live births (unpublished data). A steep increase in PRL concentrations just after discontinuation of bromocriptine in the BR method (Fig. 5C), coinciding with a physiologic PRL increase in the late luteal phase, could enhance responsiveness of selectable follicles to Gn, promoting cyclic recruitment. This might be a third mechanism by which the BR method to improve oocyte quality.

Our observations that the BR method improves embryonic quality and pregnancy rates in patients with previous ART failures were reconfirmed by 2 clinical trials in Tokushima University Hospital [103, 104] and Kitazato University Hospital [105].

Interventions affecting carbohydrate metabolism (II-3)

Glucose metabolism is essential to all human cells as a requirement for viability and biologic function, especially in terms of energy production; it is also necessary to sustain folliculogenesis. Insulin is essential to control of glucose metabolism, while insulin resistance (IR) increases oxidative stress, promoting formation of advanced glycation end-products (AGE) that induce inflammation, oxidative stress, and IR, representing vicious cycles promoting disease processes [106, 107]. Toxicity of AGE results directly from macromolecular trapping and cross-linking and indirectly from binding to AGE receptors (RAGE) [106, 108]. Vicious cycles of IR, oxidative stress, and AGE toxicity contribute to the pathogenesis of diseases such as metabolic syndrome, type 2 diabetes, hypertension, atherosclerosis, dyslipidemia, nonalcoholic fatty liver disease, central obesity, polycystic ovary syndrome (PCOS), and even conditions such as aging [106, 109–114].

IR is strongly associated with aging [115], stress [116], depression [117], obesity [118], and sedentary lifestyle [119]. These conditions are frequent among infertile women, suggesting a pathogenetic role of IR even in infertile women without PCOS. Metformin, an insulin sensitizer, consistently improves pregnancy rates in not only patients with PCOS [120] but also non-PCOS patients with repeated past failures of ART [121]. Moreover, AGE are linked to causes of infertility including PCOS, ovarian dysfunction, diminished ovarian reserve, ovarian aging, endometriosis, and male infertility [122–125]. Accordingly, the pathophysiology of infertility resembles that of other diseases involving IR.

Given the links between insulin resistance and infertility described above, we examined whether administration of low-dose metformin (500 mg/day) improved ART outcomes in non-PCOS patients with repeated ART failures in a prospective randomized controlled trial [121]. The study included infertile women who had failed to conceive in 2 or more previous ART attempts and excluded PCOS women diagnosed according to the 2003 Rotterdam criteria. Patients were allocated at random to metformin or non-metformin groups, irrespective of

assessment of insulin resistance. No patients had history of diabetes. In the same study [121], we also considered whether effectiveness of metformin can be predicted by a discriminant score (DS) calculated from multiple IR-related parameters assessed before metformin administration [121]. We administered metformin from 2 to 3 months before ART until delivery or failure, considering that glucose metabolism makes important contributions to folliculogenesis, implantation, and pregnancy. Rates of implantation and ongoing pregnancy were significantly increased by metformin in cases where the DS predicted its effectiveness (Fig. 6A).

Next, we examined correlations between AGE concentrations in blood and follicular fluid (FF) and outcomes in 157 consecutive cycles of ART [123]. Toxic AGE (TAGE) in serum, as well as pentosidine, N-carboxymethyl lysine (CML), and TAGE in FF, showed a significant negative correlation with follicular and embryonic development (Fig. 6B). When serum TAGE exceeded 7.24 U/mL, numbers of retrieved oocytes were significantly decreased early on at ages 35–40, with further decreases at ages above 40 (Fig. 6C, left panel). Attainment of ongoing pregnancy was compromised even at ages below 35 years, with greater worsening above 35 years (Fig. 6C, right panel). Even when day-3 FSH was less than 10 IU/L, likelihood of ongoing pregnancy was significantly lower in patients with TAGE exceeding 7.24 U/ml than in patients with less (6.1% *vs.* 25%).

Finally, we have improved ART outcomes by reducing AGE [126]. The water chestnut (*Trapa bispinosa* Roxb.), a common aquatic plant in Asia, Europe, and Africa that has been consumed safely as food and herbal medicine for over 2000 years, has antidiabetic, analgesic, antibiotic, and immunomodulatory activities [127]. An extract of its dried peel (Hishi extract) used as a cosmetic supplement was found to inhibit AGE formation and disrupt

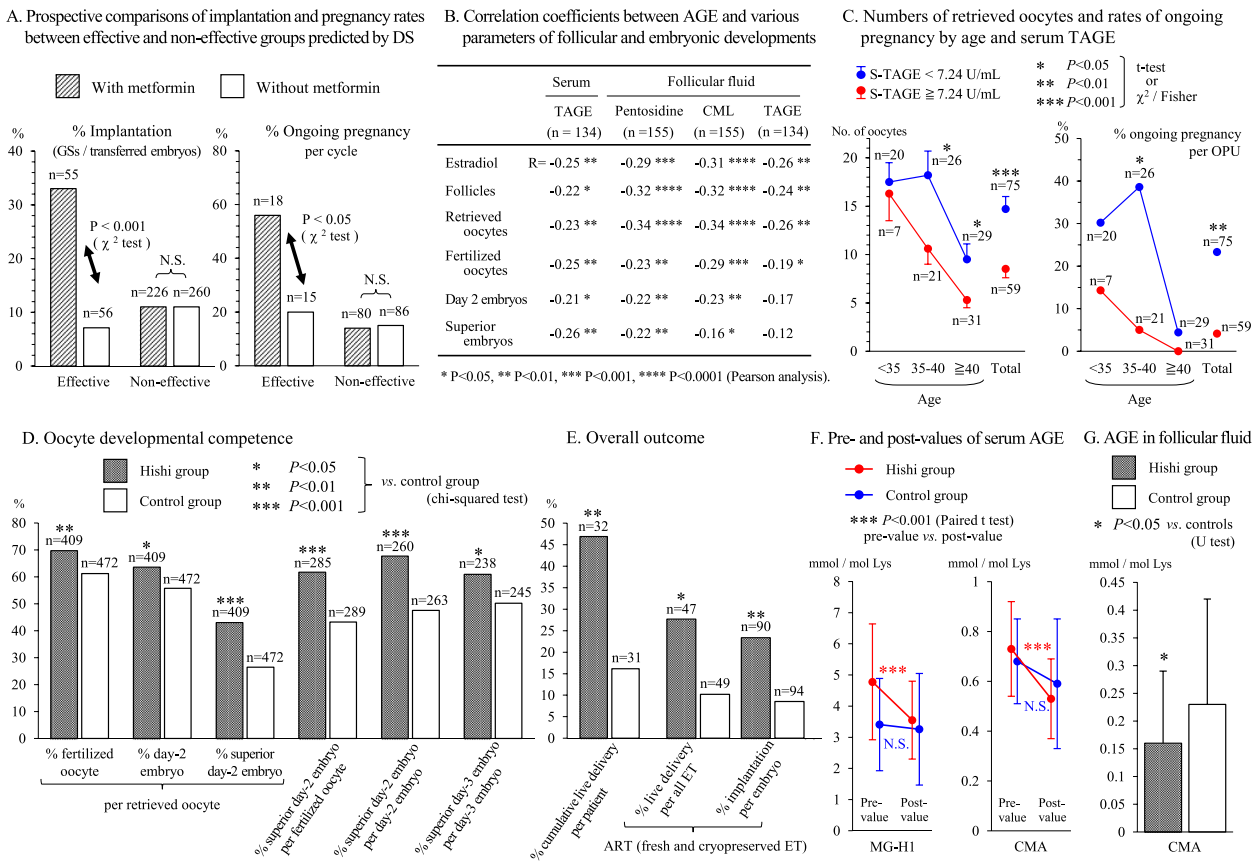

Fig. 6 Low-dose metformin increased rates of implantation and ongoing pregnancy when a discriminant score (DS), calculated from IR-related multiple parameters, predicted it as effective (**A**). Toxic AGE (TAGE) in serum and pentosidine, N-carboxymethyl lysine (CML), and TAGE in FF correlated negatively with follicular and embryonic development (**B**). Number of retrieved oocytes and rate of ongoing pregnancy decreased more sharply and from an earlier age in patients with higher TAGE in serum (**C**). Developmental potentials of oocytes and embryos (**D**), Cumulative live delivery rate per patient, live delivery rate per ET, and implantation rate (**E**) were improved by administration of Hishi extract. Hishi extract decreased N^{δ}-(5-hydro-5-methyl-4-imidazolone-2-yl)-ornithine (MG-H1) and $N.^{\omega}$-(carboxymethyl) arginine (CMA) in serum (**F**) and CMA in follicular fluid (**G**). Revised from Jinno et al., 2010 [121], Jinno et al., 2011 [123], and Jinno et al., 2021 [126]

α-dicarbonyl compounds and AGE crosslinks in vitro [128].

We conducted a prospective randomized controlled trial to determine whether Hishi extract improved ART outcome by reducing AGE [126]. The 64 study patients, with ages ranging from 38 to 42 years, were likely to have age-related AGE accumulation. In Japan, 40.7% of ART treatments involve this age group, which has a delivery rate of only 9.3% [129]; such patients are on the verge of becoming too old to overcome infertility.

The patients were randomized to groups receiving ART with or without administration of Hishi extract (100 mg/day; Pregnasupport; Hayashikane Sangyo, Shimonoseki, Japan), continuing from 2 cycles before ART until late pregnancy or failure. Both groups underwent 1 cycle of conventional infertility treatment, then 1 cycle of ART using fresh ET with a long protocol, and, if needed, cryopreserved ET until live birth or embryo depletion. Serum AGE were measured before and during ART, as were AGE in FF.

Developmental potentials of oocytes and embryos were significantly greater in the Hishi group than controls (Fig. 6D). Cumulative live birth rate among 32 Hishi patients was 47%, signifcantly higher than 16% among 31 controls ($P<0.01$; RR, 4.6; 95% CI, 1.4–15.0; Fig. 6E); 1 control patient dropped out. In ART including both fresh and cryopreserved ET, the live delivery rate per ET and implantation rate per embryo both were significantly higher in the Hishi group than in controls (28% *vs.* 10%, $P<0.05$ and 23% *vs.* 8.5%, $P<0.01$; Fig. 6E). Among 4 major fertility-related variables (age, day-3 FSH, AMH, and Hishi), only Hishi significantly correlated with cumulative live delivery *(*$P<0.05$; odds ratio, 5.1; 95% CI., 1.4–18.3; logistic regression). Fifteen Hishi and five control patients respectively delivered 17 and 5 normal live infants, including 2 and 0 sets of twins. No significant difference was evident in body weight or gestational age at delivery. No adverse effects of Hishi were observed.

Serum concentrations of N^{δ}-(5-hydro-5-methyl-4-imidazolone-2-yl)-ornithine (MG-H1) and N^{ω}-(carboxymethyl) arginine (CMA) fell significantly in the Hishi group but not in controls (Fig. 6F). Hishi extract also significantly decreased CMA in FF (Fig. 6G). In addition, reduction of AGE by Hishi administration correlated with improved folliculogenesis, fertilization, embryonic development, and implantation [126]. It is suggested, therefore, that AGE decreases as a therapeutic mechanism underlying fertility enhancement by Hishi.

How Hishi decreases AGE in vivo is much less clear. Polyphenols from water chestnut (*Trapa japonica*) husk were found to inhibit α-amylase and α-glucosidase, reducing blood glucose and insulin concentrations in mice [130]. Our study [126], however, showed little effect of Hishi extract on such glucose metabolism metrics as fasting plasma glucose, serum insulin, HOMA-R, insulinogenic index, hemoglobin A1c, glycoalbumin, C-peptide, and OGTT. Therefore, Hishi extract appeared unlikely to decrease AGE indirectly by reductions in blood glucose or enhanced insulin sensitivity or secretion. More likely, Hishi extract directly inhibited production of AGE and their intermediates or disrupted their bridging structures, as has been demonstrated in vitro [128]. MG-H1 and CMA, which are readily generated in vitro from the α-dicarbonyl compounds, methylglyoxal [131] and glyoxal, respectively [132], were particularly sensitive to the in vivo effects of Hishi in our study, which inhibits production of α-dicarbonyl compounds and degrades them in vitro [128].

Physicians should encourage patients to adopt as healthy lifestyle as possible before any interventions are attempted. Insulin sensitivity is determined physiologically by heredity, age, and lifestyle, among which only the last factor can be modified. Several points of lifestyle modification are emphasized in our clinic: (a) Be happy. Even pretending to be happy is effective. Don't worry about infertility! Worrying about anything worsens IR and various disorders, including infertility. (b) Walk continuously for more than 45 min every day, even on holidays, except when ill. Mild to moderate muscle training exercise also is recommended. (c) Go to sleep before 11 pm, wake up around 6 am, having slept for 7–8 h. (d) Adjust BMI to between 19 and 23. (e) Absolutely no smoking. (f) If you take an alcoholic drink, abstain for the following 3 days. Daily drinking of even small amounts increases IR. (g) Partake of healthy food and beverages. Ordinary meals should be freshly cooked using healthful ingredients. Avoid sugar, especially sweet drinks. Preserved food can contain AGE, which accumulate in the body if frequently consumed. Eat at a relaxed pace and chew your food.

Lifestyle modification is essential for optimum treatment even though it is often difficult. Medical interventions cannot achieve desired effects without adequate attention to lifestyle.

Danazol priming (II-4)

Danazol, a weak androgenic agent used to treat endometriosis, has been reported to improve pregnancy rate in patients with ART failures despite more than one embryo having optimal morphology [133, 134]. The original protocol involved administration of danazol at 400 mg/day for 12 weeks. ART was repeated within 3 months of the first ovulation after danazol discontinuation. We have shortened the danazol priming protocol as follows. Danazol (Bonzol; Tanabe Co., Ltd., Tokyo, Japan), 200 mg/

day, is administered for 6 weeks beginning on cycle day 2–3, followed directly by one Kaufmann cycle and then ART. For about 25 years, we have been using our danazol priming protocol successfully to overcome repeated ART failures, irrespective of embryo quality in previous ET. In this opportunity reviewing interventions affecting basal follicular growth, we decided to analyze retrospectively our ART data for the last 15 years to determine whether danazol priming improved embryo development and live birth rate as well as the likely mechanism of action. Unfortunately, we have been using this protocol simply as a routine clinical treatment without interest in researching it further, so we have not measured hormones before and after danazol administration.

One hundred thirteen relatively old patients (age: 40.8 ± 3.7 SD) with repeated ART failure (6.1 ± 4.8) underwent danazol priming and ART using a long/antagonist protocol (danazol group; 139 cycles of ART). The same 113 patients also underwent 616 cycles of ART with similar ovarian stimulation but no danazol priming (control group). ART outcomes were compared between danazol and control groups and also between subjects with and without endometriosis in the danazol group. Body weight and % body fat were measured based on electrical resistance (Body fat monitor, HBF-306, Omron Healthcare Co., Ltd., Kyoto, Japan) before and after danazol priming.

In danazol and control groups, numbers of retrieved oocytes (8.4 ± 7.1 *vs.* 7.6 ± 7.1) and fertilized oocytes (5.3 ± 4.5 *vs.* 4.6 ± 4.1) were not significantly different, but numbers of day-2 embryos (4.8 ± 4.1 *vs.* 4.1 ± 3.8) and day-3 embryos (5.5 ± 4.0 *vs.* 4.3 ± 3.4) were significantly higher in the danazol group. The rate of blastocyst acquisition per cycle was significantly higher in the danazol group than in controls (41% *vs.* 29%). Rates of clinical pregnancy (19% *vs.* 6.5%) and live birth (10.4% *vs.* 2.3%) per cycle both were significantly higher in the danazol group than in controls ($P < 0.001$; odds ratio, 3.3 and 4.9, respectively). Comparing danazol cycles in patients with (20 cycles) and without endometriosis (119 cycles), no significant differences were apparent in fertilization, embryonic development, or rates of clinical pregnancy and live birth. Danazol priming significantly increased body weight (55.1 ± 7.5 kg and 57.1 ± 7.6 before and after danazol, respectively; $P < 0.001$, $n = 107$, paired *t*-test) and decreased percent body fat (28.0 ± 4.4% and 27.5 ± 4.4 before and after danazol, respectively; $P < 0.05$, $n = 101$, paired *t*-test), suggesting an increase in amount of muscle.

Thus, danazol priming improved embryo development, pregnancy rate, and live birth rate in older women with repeated ART failure. Efficacy did not vary with presence or absence of endometriosis. The mechanism underlying the differences might be improvement of insulin sensitivity related to increased muscle mass. Alternatively, androgenic effects of danazol have enhanced preantral/small antral follicle growth, improving oocyte quality.

Danazol enhances telomerase activity in vitro [135] and has been used to treat bone marrow failure syndromes with genetic defects in telomere maintenance and repair, increasing both telomere length and peripheral-blood cell counts [136]. Recently, a randomized clinical pilot trial attempted to improve telomeric and fertility parameters in patients with diminished ovarian reserve by 3 months of danazol administration, directly followed by ovarian stimulation for ART without a connecting Kaufmann cycle– differently from our danazol priming protocol [137]. All 5 patients in their danazol group canceled their post-treatment ART due to poor follicular growth, although their blood examinations suggested an influence of danazol on telomere maintenance [137]. A few months after danazol cessation, however, the danazol patients showed a tendency for higher frequency of mature oocytes in repeated ART, compared with their ART before the danazol trial [137]. Taken together with our results, this suggests that an interval of a few weeks between danazol cessation and hMG initiation are necessary to avoid adverse effects of danazol on folliculogenesis. Further, to maximize beneficial effects of danazol priming, ovarian stimulation probably should be added at an appropriate time–meaning not too late. Consistently, danazol treatment alone without ovarian stimulation did not accomplish any improvement of live birth rate in women with unexplained subfertility, even in long term follow-up [138].

Androgen production begins with the large secondary follicle [2], increasing gradually during small antral folliculogenesis (10 weeks). Aromatase activity quickly increases from early- to mid-luteal phase [16], during the last 1–2 weeks of small antral folliculogenesis before cyclic recruitment (prerecruitment period) (sections I-3 and I-4, Fig. 1). Thus, the period of prerecruitment plus recruitment is the transitional period from androgenic to estrogenic milieu. GS-specific-androgen-receptor-knockout mice exhibited a block in folliculogenesis at the preantral secondary follicle [139], suggesting a critical role of androgen in small antral folliculogenesis. Conceivably, the first two-thirds of small antral folliculogenesis (about 6.7 weeks) simply in an increasingly androgenic milieu may safely benefit from androgen augmentation, but the last third of small antral folliculogenesis before cyclic recruitment (3.3 weeks) in the transitional period from androgenic to estrogenic milieu may be too sensitive or vulnerable to steroid manipulation. Thus, our danazol priming protocol, consisting of danazol priming

for 6 weeks, Kaufmann cycle for 3–4 weeks, and then ovarian stimulation, probably suits the changing supportive demands of small antral folliculogenesis according to developmental stage.

Other strategies (II-5)

Various pretreatments using androgens such as testosterone or dehydroepiandrosterone (DHEA), androgen-modulating agents including letrozole, recombinant LH, and hCG, pretreatment pituitary suppression using a GnRH antagonist, an estrogen, or oral contraceptive pills, GH, and coenzyme Q10, have been used to overcome poor ovarian response. Recently, Orvieto reviewed those pretreatments, concluding that none of them showed adequately convincing benefit [61].

A meta-analysis [140] including 3 randomized controlled trials (RCT) in 2006, 2009, and 2011 and one RCT in 2021 [141] showed significant increases in rates of clinical pregnancy and live birth with transdermal testosterone pretreatment in poor ovarian responders, while 3 recent RCTs in 2016, 2021, and 2023 showed no significant increases in numbers of retrieved and mature oocytes as well as clinical pregnancy rates [142–144]. Pretreatment with dehydroepiandrosterone (DHEA) in poor responders also significantly increased rates of clinical pregnancy and live birth in a meta-analysis [145] consisting of 21 studies in 2006 to 2015 (including 8 RCTs) and a retrospective cohort study in 2018 [146], whereas a recent RCT including a large number of participants (821 women) showed no beneficial effects [147]. Thus, despite the vast amount of literature on the use of testosterone and DHEA in poor responders, the bulk of evidence is still too limited to draw definite conclusions [148].

As I described in section II-4, small antral folliculogenesis within 1–2 weeks before cyclic recruitment may be vulnerable to steroid manipulation since this is a period of transition from an androgenic to an estrogenic follicular milieu. In all studies for testosterone and DHEA pretreatment up to now, androgen pretreatment was always followed directly by ovarian stimulation with no intervening interval, making follicles recruited by Gn spend the vulnerable period of small antral folliculogenesis under androgen manipulation. This adverse effect during prerecruitment period could counteract possible beneficial effects on earlier small antral folliculogenesis, leading to limited ART outcomes.

Recently, new therapeutic options such as intraovarian injection of platelet-rich plasma [149], in vitro activation (IVA), and drug-free IVA therapies [150] have been developed for patients with premature ovarian insufficiency, diminished ovarian reserve, and resistant ovary syndrome, resulting in successful pregnancies and births. While these clinical trials report promising results, such therapies require a surgical approach. Further, understanding of their efficacy, safety, and mechanism remains limited. Ongoing fundamental and clinical research is needed before wider application of these therapies.

Conclusion

About 33 years ago I repeatedly used the same long protocol in 304 patients. The pregnancy rate sharply dropped after the second attempt, and the cumulative pregnancy rate plateaued at 35% (Fig. 7A). Since then, I have developed several new methods of ovarian stimulation, as described in this review, so that pregnancy rate was improved even in patients with repeated ART failures. Naturally, different patients require different ways to enhance folliculogenesis. Although various conventional methods for ovarian stimulation are in use, the mechanism appears to be similar, involving an increase of Gn levels during folliculogenesis after cyclic recruitment. On the other hand, the high or low AMH values often encountered in infertility reflect pathogenesis involving Gn-responsive but Gn-independent folliculogenesis before cyclic recruitment (Fig. 7B). Therefore, we need to accomplish ovarian stimulation for folliculogenesis before cyclic recruitment, using means other than Gn enhancement.

Serendipitous discovery of G-CSF priming taught me that modulation of basal follicular growth is feasible, encouraging me to seek new ways for the BR method to improve folliculogenesis since BR has helped so many patients in my ART practice over 3 decades. I believe that the steep increase in PRL that immediately follows discontinuation of bromocriptine is likely to enhance responsiveness of selectable follicles to Gn for cyclic recruitment. Consistently in our recent pilot study, increased PRL in the late luteal phase mimicked beneficial effects of the BR method on oocyte quality. Also, interventions affecting glucose metabolism, such as increasing insulin sensitivity with metformin and decreasing AGE with Hishi extract, work best when started 1 to 2 cycles before the ART cycle, i.e., during early antral folliculogenesis. Danazol, a weak androgen, administered during the first two-thirds of that time improves oocyte developmental competence and live birth rate.

Without question, the 4 methods just discussed need to be validated by further investigations. I have published and presented the BR method in journals and scientific meetings, getting limited attention because of a stereotype that "increasing PRL is bad" as well as unawareness of the importance of extrapituitary PRL and PRL in general. For 3 decades, however, I still have successfully used these methods in my clinic because they work

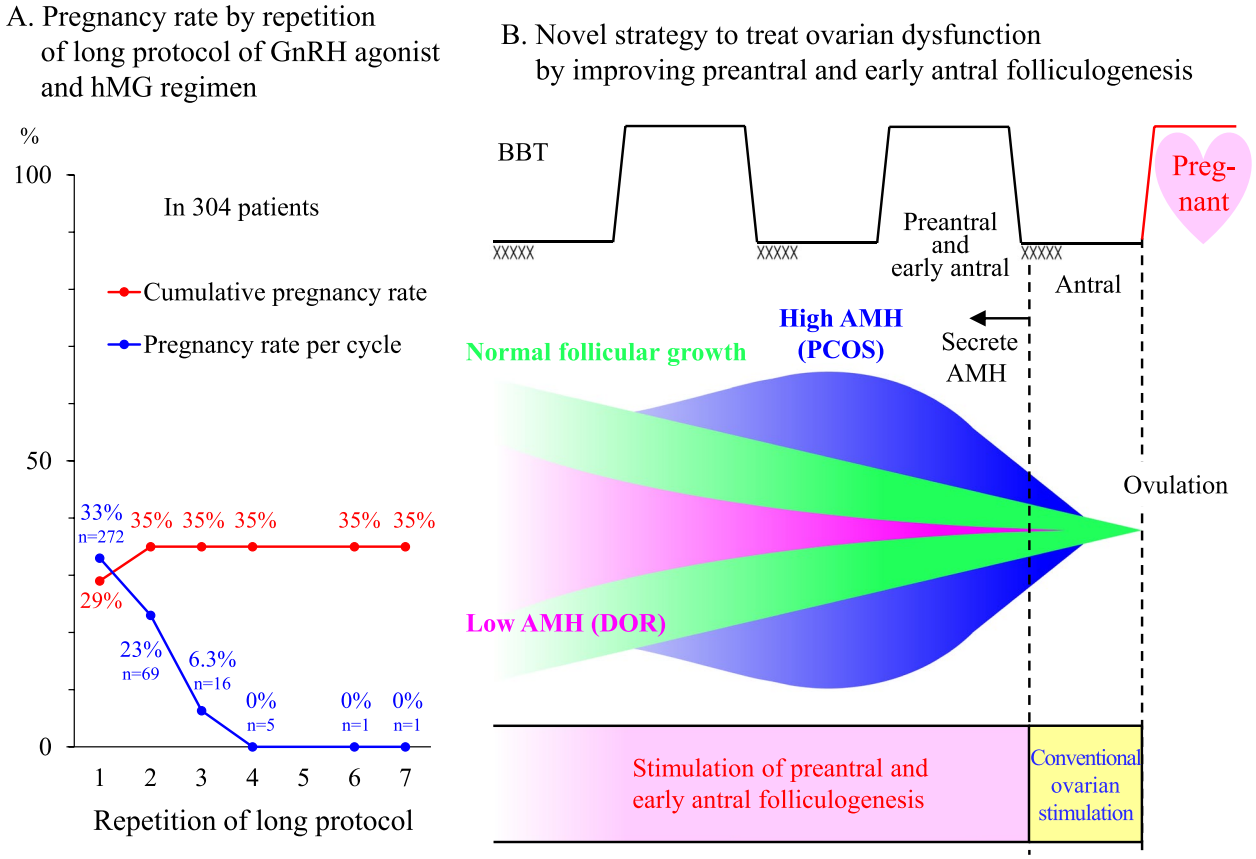

Fig. 7 Pregnancy rate steeply decreased while repeating the same protocol of ovarian stimulation (**A**). A novel strategy to treat ovarian dysfunction by improving preantral and early antral folliculogenesis (**B**)

dependably in my patients, enabling thousands of births. I believe that time will bear me out.

The methods explained in this review act independently of Gn, improving early antral folliculogenesis before cyclic recruitment, although the BR method, metformin, and Hishi extract additionally enhance later antral folliculogenesis. I hope that these treatments will help many more patients who are refractory to conventional ovarian stimulation, and that basic researchers and physicians will further explore stimulation of preantral and early antral folliculogenesis.

Abbreviations

AGE	Advanced glycation end-products
AKT	Protein kinase B
AMH	Anti-Müllerian hormone
ART	Assisted reproductive technology
BDNF	Brain-derived neurotrophic factor
BFGF	Basic fibroblast growth factor
BIRC	Baculoviral inhibitors of apoptosis repeats-containing proteins
BMI	Body mass index
BMP	Bone morphogenetic protein
BMSC	Bone marrow stem cells
BR method	Bromocriptine-rebound method
CCN	Cysteine-rich angiogenic protein 61, connective tissue growth factor, and nephroblastoma overexpressed
CI	Confidence interval
CMA	N^{ω}-(carboxymethyl) arginine
CML	N-carboxymethyl lysine
CNP	C-type natriuretic peptide
DHEA-S	Dehydroepiandrosterone sulfate
DOR	Diminished ovarian reserve
EGF	Epidermal growth factor
ET	Embryo transfer
E_2	17β-Estradiol
FOXO3	Forkhead box O3
FSH	Follicle-stimulating hormone
GC	Granulosa cell(s)
G-CSF	Granulocyte colony-stimulating factor
GDF	Growth differentiation factor
Gn	Gonadotropin
GnRH	Gonadotropin-releasing hormone
hCG	Human chorionic gonadotropin
HGF	Hepatocyte growth factor
hMG	Human menopausal gonadotropin
ICSI	Intracytoplasmic sperm injection
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IR	Insulin resistance
IVA	in vitro activation
IVF	in vitro fertilization
IWI	Interwave interval
JAK/STAT	Janus kinase /signal transducer and activator of transcription

KGF	Keratinocyte growth factor
KIR	Killer-cell immunoglobulin-like receptor
LATS1/2-Mob1	Large tumor suppressor homolog 1/2-MOB kinase activator 1
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
MAPK	Mitogen-activated protein kinase
MG-H1	N^{δ}-(5-hydro-5-methyl-4-imidazolone-2-yl)-ornithine
MST1/2-Sav1	Mammalian Ste-20 like kinase 1/2-Salvador 1
mTORC1	Mammalian target of rapamycin complex 1
NPR2	Natriuretic peptide receptor 2
NTF5	Neurotrophin 5
OPU	Oocyte pick-up
OS	Ovarian stimulation
PAPP-A	Pregnancy-associated plasma protein-A
PCOS	Polycystic ovary syndrome
PDK1	Phosphatidylinositol-dependent kinase 1
PETEN	Phosphatase and tensin homolog
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol-4,5-biphosphate
PIP_3	Phosphatidylinositol-3,4,5-triphosphate
PRL	Prolactin
PRP	Platelet-rich plasma
RAGE	AGE receptor
RCT	Randomized controlled trial
RIF	Repeated implantation failure
rpS6	Ribosomal protein S6
RR	Relative risk
RSOS	Random start ovarian stimulation
SD	Standard deviation
S6K1	P70 S6 kinase 1
TAGE	Toxic AGE
TEAD	Transcription factors containing the TEA (transcriptional enhancer activator) DNA binding domain
TIC	Theca interna cell(s)
TGF	Transforming growth factor
TKR	Tyrosine kinase receptor
TSC1/2	Tuberous sclerosis complex 1/2
VEGF	Vascular endothelial growth factor
WNT	Wingless
YAP/TAZ	Yes-associated protein/transcriptional coactivator with PDZ-binding motif

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Author's contributions
Masao Jinno, MD, PhD made almost all contributions to study design and to acquisition, analysis, and interpretation of data. He wrote all part of this article. He gave final approval for this version of the manuscript to be published.

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Data availability
No datasets were generated or analysed in the part of review in this study. The datasets used and/or analyzed in the section of II-4. danazol priming in this study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Informed consent was obtained from all patients. The study was approved by the Ethics Committees of Women's Clinic Jinno. Clinical trials referenced in this review were registered with the UMIN in Japan and each registration number was stated in each published paper. Our research was conducted in accordance with the Declaration of Helsinki.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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